Attorney Docket No. 035718/208255

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Duvick *et al.*	Confirmation No.:	1574
Appl. No.:	09/882,694	Group Art Unit:	1638
Filed:	June 15, 2001	Examiner:	M.A. Ibrahim
For:	COMPOSITIONS AND METHODS FOR FUMONISIN DETOXIFICATION		

April 20, 2004

Assistant Commissioner for Patents
Washington, DC 20231

RULE 132 DECLARATION
of
Jon Duvick

Sir:

I, Jon Duvick, do hereby declare and say as follows:

1. I am skilled in the art of the field of the invention of the above-referenced application. I have a Ph.D in Plant Pathology from the University of Wisconsin-Madison, USA. Since 1977, I have been engaged in the study of plant disease resistance. I have been employed by Pioneer Hi-Bred since 1984 and have worked in the area of fumonisin degradation for 10 years.

2. I have read and understood the specification and claims of this application and have read and understood the Office Action in this case dated January 30, 2004 ("Office Action").

3. The Office Action (page 3, first paragraph) rejected claims 1-33 as "failing to comply with the enablement requirement." The Office Action states (page 4, first paragraph) as a basis for the enablement rejection that "the reaction catalyzed by microbial esterase or amine oxidase may be reversible, and might have therefore failed to protect plant cells from the fumonisin and/or AP1." I disagree with this statement, because the

reactions mentioned are not likely to be reversible under normal pH levels and substrate concentrations.

4. The Office Action states (page 4, first paragraph) as a basis for the enablement rejection that "it is uncertain how the metabolism of the reaction products (for example, 2-OP and ammonia) might affect the plant.... It is also uncertain, even if fumonisin produced by an invading fungus was efficiently converted to a non-toxin form, whether this conversion would impart reduced fumonisin toxicity upon the plant." I disagree with this statement. The metabolic product 2-OP has been demonstrated to be nontoxic with respect to sphingolipid metabolism and has been shown to be nonmutagenic in a preliminary Ames test. Further, 2-OP shows no similarity to known toxins, so there is no reason to believe it would be toxic, and no reason to believe that it would adversely affect a plant.

5. The Office Action states (page 4, first paragraph) as a basis for the enablement rejection that "[i]t is also uncertain, even if fumonisin produced by an invading fungus was efficiently converted to a non-toxin form, whether this conversion would impart reduced fumonisin toxicity upon the plant." I don't understand the rationale for this statement, because converting a compound to a non-toxic form would necessarily result in reducing the toxicity of that compound to a plant. In fact, the present specification provides working examples which demonstrate that the metabolism of fumonisin to AP1 resulted in at least a 30-fold decrease in toxicity to plant tissues and as much as a 100-fold decrease in toxicity to plant cells (see Example 3, page 36, and Example 4, page 37).

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: April 22, 2004 By: _____

Jon Duvick

5.4.3

EXPRESSION OF OXALATE OXIDASE IN SUNFLOWER TO COMBAT *SCLEROTINIA* DISEASE

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Background and objectives

Resistance to the fungal pathogen *Sclerotinia* is a trait of major importance for crops such as sunflower, canola, and soybean. Sunflower *Sclerotinia* disease can be established at various developmental stages, with the main targets being head, stem and root tissues [1]. This suggests that resistance genes need to be constitutively expressed in multiple tissues. The major toxic and pathogenic factor produced by *Sclerotinia* is oxalic acid that can be converted into H_2O_2 and CO_2 by oxalate oxidase.

Results and conclusions

A candidate gene for detoxifying oxalate is the wheat oxalate oxidase which has been used to transform a sunflower inbred line. Our greenhouse and field trials demonstrated that expression of this oxalate oxidase by constitutive promoters significantly enhances resistance to *Sclerotinia* in sunflower.

References

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Engineered detoxification confers resistance against a pathogenic bacterium

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We generated transgenic sugarcane plants that express an albicidin detoxifying gene (*albD*), which was cloned from a bacterium that provides biocontrol against leaf scald disease. Plants with albicidin detoxification capacity equivalent to 1–10 ng of AlbD enzyme per mg of leaf protein did not develop chlorotic disease symptoms in inoculated leaves, whereas all untransformed control plants developed severe symptoms. Transgenic lines with high AlbD activity in young stems were also protected against systemic multiplication of the pathogen, which is the precursor to economic disease. We have shown that genetic modification to express a toxin-resistance gene can confer resistance to both disease symptoms and multiplication of a toxigenic pathogen in its host.

Keywords: disease resistance, antipathogenesis, transgenic plants, sugarcane, albicidin detoxification

Many pathogenic bacteria produce toxins that induce symptoms characteristic of the associated disease in their animal or plant hosts[1,2]. These toxins are not the primary determinants of host range, as sometimes occurs with fungal phytotoxins in plants[3]. However, bacterial phytotoxins may be primary determinants of disease by suppressing host resistance mechanisms[4] to permit systemic multiplication by the pathogen[5], in addition to causing the economically damaging symptoms. There are indications that genetic modification for increased resistance to toxins involved in pathogenesis may be developed as a novel mechanism of disease resistance[6–8], but it has not been possible to test the approach against a systemic pathogen in its host species.

Leaf scald disease of sugarcane (*Saccharum officinarum*, polyploid interspecific hybrids) is a finely balanced host–pathogen interaction, with prolonged latent infection often preceding the development of damaging chronic symptoms or the devastating acute phase of the disease[9]. The systemic, xylem-invading pathogen (*Xanthomonas albilineans*) produces a family of low molecular weight toxins (albicidins) that selectively block prokaryote DNA replication[10] and cause the characteristic chlorotic symptoms by blocking chloroplast development[11,12]. Tox$^-$ mutants and transgenic *X. albilineans* strains expressing a novel gene for albicidin detoxification are attenuated in pathogenicity[13–15], indicating that albicidins also may be a key factor in systemic invasion or the unpredictable transition from latent infection to disease.

We used the highly efficient genetic transformation system for sugarcane[16] and the recently cloned *albD* gene for albicidin detoxification[15] to explore engineered inactivation of pathogenesis factors as a control strategy against bacterial diseases. We report that expression in transgenic sugarcane of a novel gene for albicidin detoxification can confer a high level of resistance to chlorotic symptom induction, and multiplication and systemic invasion by *X. albilineans*.

Results and discussion

Production and challenge of transgenic plants. Transgenic plants of leaf scald–susceptible sugarcane cultivars Q63 and Q87 were regenerated following particle bombardment into embryogenic callus and selection for G418 resistance (conferred by *aphA* expression). The *albD* gene, controlled by the maize *ubi* promoter, was coprecipitated with a selectable *aphA* construct onto the microprojectiles before bombardment. After three months in the greenhouse, 36 independent transformants from cultivar Q63, 25 transformants from cultivar Q87, and 22 known AlbD$^-$ controls were challenged with *X. albilineans* by a decapitation method that simulates the spread of this mechanically transmitted pathogen in the field. Two weeks after inoculation, all plants of the AlbD$^-$ control lines (untransformed or transformed with genes other than *albD*) showed typical white pencil-line symptoms. In contrast, 44% (Q63) to 56% (Q87) of independent transformants from bombardments including *albD* showed no symptoms in inoculated leaves (Fig. 1). Identical results were obtained in the same plant lines inoculated using either of two *X. albilineans* isolates or juice from leaf scald–diseased sugarcane.

PCR tests for the *albD* gene were positive for 90% of the transformants from bombardments including *albD*, and 75% had *albD* transcripts detectable in young leaves by northern blot analysis. This high coexpression frequency for selected and unselected genes coprecipitated on separate plasmids is consistent with previous results using reporter genes in the sugarcane transformation system[16]. There was a highly significant correlation between *albD* transcript levels and AlbD enzyme activity in young leaves of 24 tested transformants (Spearman's rank correlation coefficient $r = 0.79$, $P < 0.0001$, Fig. 2). The *ubi* promoter is strongly inducible by various biotic and abiotic stresses[17]. Sugarcane *ubi-luc* transformants show spatial and temporal variation in transgene activity within lines, as expected because of microenvironmental effects on inducible promoter activity[18]. Some scatter in the relationship between mRNA and gene product levels from an inducible promoter such as *ubi* is expected, depending on the relative stabilities of the mRNA and protein and the sampling time relative to stresses that transiently enhance transcription[19]. Therefore, we measured AlbD enzyme activity in experiments to test directly the relationship between level of the active transgene product and protection against leaf scald disease.

Effect of AlbD activity on chlorotic symptom development. One month after inoculations, AlbD enzyme activity was measured in leaf and stem tissues of uninoculated plants of each line. There was a strong negative correlation between AlbD activity in the youngest

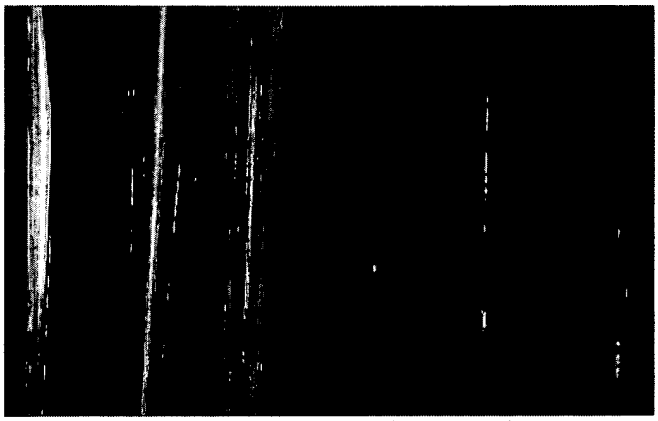

Figure 1. Leaves of sugarcane cultivar Q63 following inoculation with the leaf scald pathogen *X. albilineans*, showing characteristic white pencil-line streaks surrounding invaded vascular bundles in AlbD⁻ control lines (including untransformed, GUS transformed and LUC transformed Q63), and absence of symptoms in three independent AlbD⁺ transformed lines.

fully emerged leaf and the number of white pencil lines developed in inoculated leaves ($r = -0.48$, $P < 0.0001$). Plant lines with high AlbD activity (>200 ng albicidin detoxified per mg soluble protein) showed few or no white pencil lines. Severely diseased plant lines (more than five white pencil lines per plant) had little or no AlbD activity (Fig. 3). Albicidins are produced as a family of secondary metabolites. These metabolites may differ in relative antibacterial and phytotoxic activities, but all appear to be inactivated by AlbD enzymatic activity[15]. The *X. albilineans* strains engineered to express *albD* do not cause chlorosis in leaf scald–susceptible sugarcane[15], and sugarcane engineered to express *albD* does not develop chlorosis following inoculation with wild-type toxigenic *X. albilineans* (Fig. 3). It can be concluded that albicidins are the pathogenesis factor responsible for the damaging chlorotic symptoms of sugarcane leaf scald disease.

Effect of AlbD activity on pathogen multiplication. Although chlorosis is a spectacular visible manifestation of albicidin action in the leaves, the consequences of blocked plastid division and differentiation in other tissues exposed to the toxin during systemic colonization of the xylem by *X. albilineans* may be of greater relevance in disease development. Plastids are the site of essentially all fatty acid biosynthesis, nitrite reductase activity, essential amino acid biosynthesis, and production of essential precursors of defense-related aromatic compounds in plants[20]. It follows that albicidin-mediated disruption of plastid division and development could facilitate systemic invasion and disease development, by weakening plant defense mechanisms. We tested this possibility by comparing multiplication of the pathogen in inoculated control and *albD*-expressing sugarcane lines.

Two weeks after inoculation, *X. albilineans* had multiplied to approximately 10^6 colony-forming units per gram of fresh weight (c.f.u./g.f.w.) in inoculated leaves of all control lines. There was a strong negative correlation between AlbD activity in the youngest fully emerged leaf and pathogen multiplication in inoculated leaves ($r = -0.60$, $P = 0.009$). The *X. albilineans* population in inoculated leaves was restricted to $<5 \times 10^3$ c.f.u./g.f.w. in four of the five tested lines with >100 ng albicidin detoxified per mg soluble protein (Fig. 4A). This indicates that albicidin resistance due to *albD* expression in transgenic sugarcane can substantially reduce *X. albilineans* multiplication in the primary infection court (mechanically inoculated leaves).

Two months after inoculation, *X. albilineans* had invaded systemically to reach high populations in the midstem ($>3 \times 10^5$ c.f.u./g.f.w.

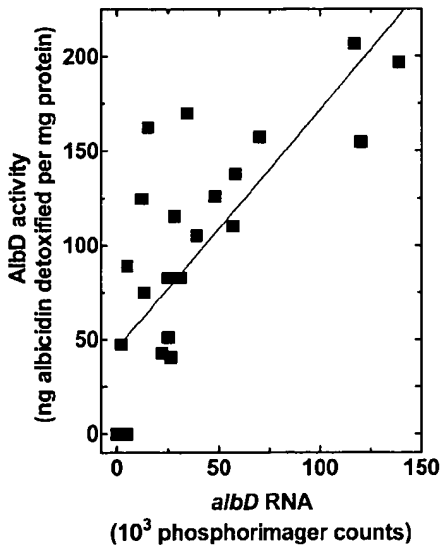

Figure 2. Relationship between *albD* mRNA levels and AlbD enzyme activity in young leaves of sugarcane cultivar Q63 and 24 independent transgenic lines grown under containment greenhouse conditions. There is a highly significant correlation between these measures ($r = 0.79$, $P < 0.0001$), with some scatter as expected from the inducible Ubi promoter.

in internodes subtending inoculated leaves) and stem apex ($>3 \times 10^4$ c.f.u./g.f.w., at least four internodes above inoculated leaves) for four out of five tested AlbD⁻ control lines. Three of the five transgenic lines with the highest AlbD activity in leaves also showed highest activity (>25 ng albicidin detoxified per mg soluble protein) in the stem apex region (2 cm above to 1 cm below the apical meristem). In these three lines, *X. albilineans* populations in the midstem and stem apex were near or below the detection limit of 10^2 c.f.u./g.f.w., a 3–4 log reduction relative to AlbD⁻ controls (Fig. 4B). The strong negative correlation between AlbD activity and pathogen multiplication observed in inoculated leaves was maintained for systemic invasion ($r = -0.37$, $P = 0.02$). We conclude that albicidin resistance in trans-

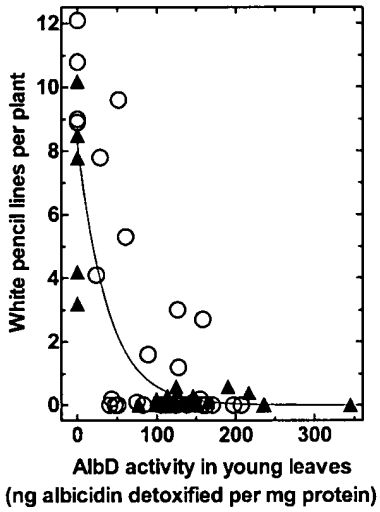

Figure 3. Relationship between albicidin detoxifying activity from expression of the *albD* transgene, and disease severity in leaves inoculated with *X. albilineans*. AlbD activity in the first fully emerged leaves is the mean from two plants per transformed line. The number of white pencil lines per plant, measured two weeks after inoculation by the decapitation method, is the mean from 10 plants per transformed line of leaf scald–susceptible sugarcane cultivars Q63 (○) and Q87 (▲). The data fit an exponential decline in disease severity with increasing AlbD activity ($R^2 = 0.71$).

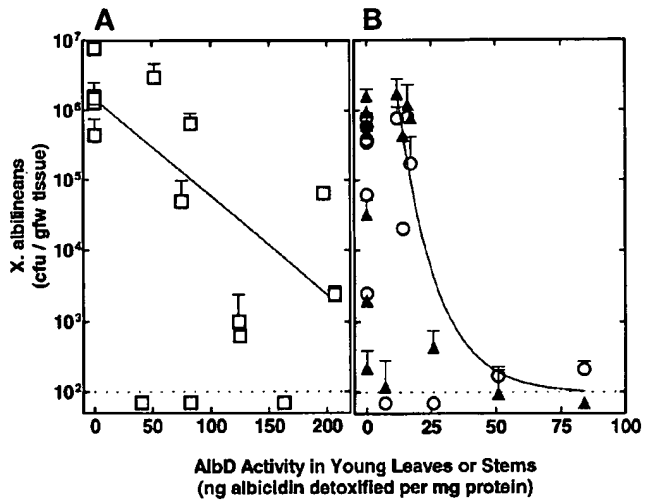

Figure 4. Relationship between albicidin detoxifying activity from expression of the *albD* transgene, and multiplication of the pathogen *X. albilineans* in inoculated plants of sugarcane cultivar Q63. AlbD activity is the mean from two plants per transformed line, measured in the first fully emerged leaves (A) or in the stem apex region (B). Pathogen populations are means with standard errors from two plants per transformed line, measured after two weeks in inoculated leaves (□), and after two months in midstem (○) and stem apex (▲) regions. Populations below the detection limit of 10^2 c.f.u./g.f.w. are shown below the dashed line at this threshold. The data for log pathogen population fits a linear decline with increasing AlbD activity in leaves (R^2 = 0.58), and an exponential decline with increasing AlbD activity above 10 ng albicidin detoxified per mg protein in stems (R^2 = 0.84).

genic sugarcane, due to expression of the *albD* gene for enzymatic detoxification, not only protects against damaging chlorotic symptoms, but can also protect the plants against systemic invasion and development of high pathogen populations in stalks.

Leaf scald disease symptoms and yield losses in the field are observed only in stalks with high pathogen populations[21]. The genetic complexity of sugarcane means that backcrossing to eliminate single undesired traits from elite cultivars is not feasible. However, the crop is clonally propagated, and an efficient genetic transformation system is applicable to most tested cultivars[16]. The AlbD enzyme activity in the sugarcane lines with the highest activity characterized here corresponds to 1–3 ng AlbD/mg soluble protein in cell extracts from the stem apex region, and 5–6 ng/mg from fully expanded leaves (based on reconstruction experiments with purified AlbD protein). The strong correlation between AlbD activity and disease resistance (Fig. 3 and 4) indicates that sugarcane transformants with 5–10 ng AlbD/mg protein in stems and young leaves should be resistant to leaf scald disease. This low level of an introduced gene product is not expected to impose any substantial metabolic load. Genetic transformation to obtain clones with an appropriate pattern of *albD* expression is therefore a promising approach to rescue agronomically outstanding sugarcane cultivars that would otherwise have to be discarded because of susceptibility to leaf scald disease.

Our results confirm that toxins first recognized because of the disease symptoms that they elicit can play a larger role in pathogenesis by rendering the host more susceptible to invasion. We have demonstrated that genetic modification to express a toxin-resistance gene can confer resistance to both disease symptoms and multiplication of a toxigenic pathogen in its host. It is likely that this antipathogenesis approach can be developed to target pathogenicity factors other than toxins—for example, signal molecules used in quorum sensing by pathogens, or compounds such as hormones and enzymes that elicit host responses favorable to the pathogen. An advantage of the approach is that, in most cases, such resistance is expected to be stable, because it can be overcome only through a gain of function by the pathogen.

Experimental protocol

Genetic constructs and transformation. The coding region of the *albD* gene from *Pantoea dispersa*[15] was cloned between the *ubi* promoter from the maize *ubi-1* gene[17] and the *Agrobacterium nos* terminator[22] to drive expression in sugarcane cells. Leaf scald–susceptible sugarcane cultivars Q63 and Q87 were transformed by coprecipitation of the *ubi-albD* construct (pU3Zald) and an *aphA* construct (pEmuKN) onto tungsten microprojectiles, bombardment into embryogenic callus, selection for resistance to antibiotic G 418 (Geneticin; GibcoBRL, Rockville, MD), and regeneration of transgenic plants[16].

Inoculations and pathogen multiplication. Five plants per transgenic line from *albD* bombardments were inoculated with *X. albilineans* isolate XA3, and five plants per line were inoculated with juice from leaf scald–diseased sugarcane. An additional five plants per Q63 line were inoculated with a fresh *X. albilineans* isolate XA15. Untransformed Q63 and Q87 regenerated from embryogenic callus culture, and Q63 lines transformed with *ubi-luc*, *ubi-uidA* or *ubi*-antisense *albD* constructs were used as known $AlbD^-$ control lines (22 in total). Inoculum (200 µl, containing 8×10^8 cells for the cultured isolates) was applied to the freshly cut surface of plants decapitated above the apical meristem. This decapitation method is widely used in field testing sugarcane varieties for resistance to the disease[9]. Inoculated leaves emerging from the cut spindle typically show characteristic white pencil-line symptoms, resulting from blocked plastid differentiation in parenchyma cells surrounding invaded vascular bundles[12]. *X. albilineans* populations were determined in tissues of five $AlbD^-$ control lines and 10 $AlbD^+$ lines of Q63, by plating extracts on selective medium as described[13,14].

AlbD enzyme assays. One-gram tissue samples were ground to a powder in liquid nitrogen, ground for an additional 1 min with 3 ml of extraction buffer (100 mM KPO_4, 1 mM EDTA, 1% Triton X-100, pH 7), kept on ice for 10 min, then centrifuged at 4°C for 20 min at 14,000 *g*. To quantify AlbD activity in the supernatant, albicidin was added to a concentration of 15 ng/µl. Reaction mixtures were incubated at 37°C for 30 min, then stopped by adding 10% SDS to a final concentration of 1%. Remaining albicidin was quantified in a plate bioassay using *Escherichia coli* DH5α as the indicator strain[10,14]. The results (presented as nanograms of albicidin detoxified per milligram of soluble protein in the plant tissue extracts) accurately rank samples for AlbD enzyme activity. The relationship to AlbD enzyme concentration is nonlinear, because it is not possible to maintain saturating albicidin (substrate) concentrations in the assay.

Northern blot analysis. RNA was extracted from young leaves of control and transgenic plants[23] at the time of sampling for AlbD enzyme assays. RNA samples (15 µg/lane) were electrophoresed in a 1.2% agarose gel containing formaldehyde, transferred to nylon membrane, and hybridized[24] using ^{32}P-labeled coding region of *albD* amplified by PCR from pQZE533 (ref. 15). Signal intensity in the band corresponding to the *albD* transcript (after excision of the *ubi* intron 1) was quantified using a Molecular Dynamics Phosphorimager SI.

Acknowledgments

We thank the Queensland Bureau of Sugar Experiment Stations for assistance with sugarcane. This work was supported by the Australian Research Council and the Sugar Research and Development Corporation.

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